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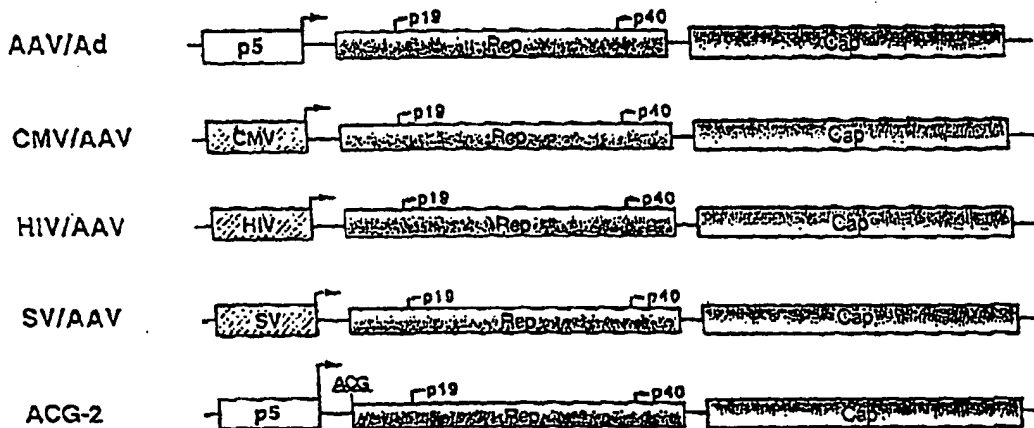
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(54) Title: METHODS FOR INCREASING THE EFFICIENCY OF RECOMBINANT AAV PRODUCT



(57) Abstract

The present invention relates to methods and compositions for increasing the production of high titre stocks of recombinant AAV (rAAV) through regulation of expression of the AAV REP and CAP proteins. The methods and compositions of the invention are based on the observation that the low level expression of the AAV REP protein increases the production of AAV viral capsid protein and efficiency of packaging resulting in production of higher titre recombinant viral stocks. The invention encompasses recombinant AAV vectors that direct the expression of AAV REP and CAP proteins (see the figure) and the use of such vectors for the production of novel stable cell lines capable of generating high titre rAAV vectors. The invention provides methods for regulating the expression of the AAV REP gene at the transcriptional and post-translational level.

METHODS FOR INCREASING THE EFFICIENCY OF
RECOMBINANT AAV PRODUCT

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1. INTRODUCTION

10 The present invention relates to methods and compositions for increasing the production of high titre stocks of recombinant AAV (rAAV) through regulation of expression of the AAV REP proteins. The methods and compositions of the invention are based on the observation that low level expression of the AAV REP protein increases
15 the efficiency of rAAV DNA replication and the production of AAV viral capsid protein resulting in production of higher titre recombinant viral stocks. The invention encompasses methods and compositions for controlling the level of REP expression at the transcriptional or translational level.
20 Additionally, the invention provides methods for regulating the biological activity and/or stability of the REP proteins at the post-translational level. The methods and compositions of the invention can be used to produce high titre stocks of rAAV which can be used in gene therapy for
25 the purpose of transferring genetic information into appropriate host cells for the management and correction of human diseases including inherited and aquired disorders.

2. BACKGROUND OF THE INVENTION

30 2.1. GENE THERAPY

Gene therapy is generally understood to refer to techniques designed to deliver functionally active therapeutic genes into targeted cells. Such therapeutic genes may encode proteins that complement genetic
35 deficiencies, cytokines, cell surface membrane proteins or any protein that functions to regulate cell growth and/or differentiation. Such proteins may function intracellularly,

for example, by regulating a signalling pathway or transcriptional pathway. Alternatively, the proteins may be secreted by the cell and exert their effect extracellularly.

Initial efforts toward somatic gene therapy have relied
5 on indirect means of introducing genes into tissues, e.g., target cells are removed from the body, transfected or infected with vectors carrying recombinant genes, and reimplanted into the body. These types of techniques are generally referred to as in vitro treatment protocols.

10 In addition, recombinant replication-defective viral vectors have been used to infect cells both in vitro and in vivo. Perhaps the most widely studied viral vectors for use in gene therapy have been the retroviral vectors. The major disadvantages associated with the use of retroviral vectors
15 include the inability of many viral vectors to infect non-dividing cells, problems associated with insertional mutagenesis and potential helper virus production. Recently, attention has turned to the use of other types of recombinant viral vectors such as adenovirus and adeno-associated virus
20 based vectors, that may be used to deliver genes of interest to cells.

In particular, recombinant adeno-associated virus has many features of interest in the field of gene therapy. The vectors are based on a defective, nonpathogenic human
25 parvovirus that can infect both dividing and non-dividing cells without a marked tropism. In addition, the viral genome can stably integrate within the host genome, facilitating long term gene transfer.

30 2.2. AAV VIRAL VECTORS

The AAV genome is composed of a linear single stranded DNA molecule of 4680 nucleotides which contains major open reading frames coding for the Rep (replication) and Cap (capsid) proteins. Flanking the AAV coding regions are two
35 145 nucleotide inverted terminal (ITR) repeat sequences that contain palindromic sequences that can fold over to form hairpin structures that function as primers during initiation

of DNA replication. In addition, the ITR sequences are needed for viral integration, rescue from the host genome and encapsidation of viral nucleic acid into mature virions (Muzyczka, N., 1992, Current Topics in Microbiology & Immunology. 158, 97-129).

AAV can assume two pathways upon infection into the host cell depending on whether helper virus is present. In the presence of helper virus, AAV will enter the lytic cycle whereby the viral genome is transcribed, replicated, and encapsidated into newly formed viral particles. In the absence of helper virus function, the AAV genome will integrate as a provirus into a specific region of the host cell genome through recombination between the AAV termini and host cell sequences (Cheung, A. et al., 1980, J. Virol. 33:739-748; Berns, K.I. et al., 1982, in Virus Persistence, eds. Mahey, B.W.J., et al. (Cambridge Univ. Press, Cambridge), pp. 249-265).

The use of AAV as a vehicle for the transfer of genetic information has been facilitated by the discovery that when a plasmid containing an intact AAV genome is transfected into a host cell the recombinant AAV vector will integrate into the host cell genome and remain as a provirus until the host cell subsequently becomes infected with a helper virus. Upon infection of the host cell with helper virus, the AAV is rescued out from the plasmid vector and enters the lytic cycle leading to production of mature virions.

The production of rAAV particles, utilizes a vector containing a transgene flanked by the inverted terminal repeats (ITR), which are the sole AAV cis sequences required for DNA replication, packaging and integration. To produce rAAV particles, the AAV (Rep) and capsid (Cap) gene products are normally provided in trans from a different template, usually a helper plasmid.

The three viral coat proteins, VP1, VP2, and VP3 which are required for virion expression are derived from mRNA initiated at the p40 promoter, while the four overlapping non-structural Rep proteins are essential for AAV DNA

replication. Rep78 and 68 are expressed from unspliced and spliced transcripts initiating at the p5 promoter, while Rep52 and Rep40 are similarly produced from transcripts initiating at the p19 promoter. Although Rep52/40 have been implicated in AAV single stranded DNA formation (Chejanovsky et al., 1989, Virology 173:120-128) and gene regulation, Rep appear to display all enzyme functions essential for AAV DNA replication, (ITR binding, DNA helicase, and DNA site-specific nicking activity), (Muzyczka, N., 1991, Seminars in Virology 2:281-290). In addition to these functions, Rep both positively and negatively regulate AAV promoters (Labow et al., 1986, Journal of Virology 60:215-258; Pereira et al., 1997, J. Virol, In Press; Tratschin et al., 1986, Mol. Cell Biol. 6:2884-2894) and repress numerous heterologous promoters (Antoni et al., 1991, Journal of Virology 65:396-404; Heilbronn et al., 1990, Journal of Virology 64:3012-3018; Hermonat, P.L., 1994, Cancer Letters 81:129-36; Horer, et al., 1995, Journal of Virology 69:5485-5496; Labow et al., 1987, Molecular & Cellular Biology 7:1320-1325).

Rep gene expression appears to be critical for all steps of the AAV life cycle, including a latent state which occurs in the absence of a helper virus (Berns, K.I., 1990, Virology, 2ed, vol. 2; Berns, K.I., 1996, B.N. Fields et al. ed.; Samulski et al., 1989, Journal of Virology 63:3822-3828). Recently, Rep have also been associated with AAV site-specific integration (Giraud et al., 1994, Proceedings of the National Academy of Sciences of the United States of America; Kotin et al., 1990, Proceedings of the National Academy of Sciences of the United States of America 87:221-2215; Samulski et al., 1991, EMBO Journal 10:3941-3950; Weitzmann et al., 1994, Proceedings of the National Academy of Sciences of the United States of America 91:5808-5812). Repression of viral gene expression by rep and host YY1 protein appears to be required for establishment and maintenance of the latent state (Labow et al., 1986, Journal of Virology 60:251-258; Laughlin et al., 1982, Journal of Virology 41:868-876; Pereira et al., 1997, J. Virol In Press;

Shi et al., 1991, Cell 67:377-388). Such repression may be necessary to avoid the demonstrated cytostatic effect on the host cell by Rep gene products (Yang et al., 1994, Journal of Virology 68:4847-4856). During a lytic infection, the AAV 5 promoters, particularly p5, are transactivated by the adenovirus E1A proteins and YY1 (Lewis, et al., 1995, J. Virol. 69:1628-1636; Shi et al., 1991, Cell. 67:377-388). The p5 products then positively regulate the p19 and p40 promoters, resulting in abundant production of Rep 52/40 and 10 viral capsid proteins (Pereira et al., 1997, J. Virol. In Press). Early effort to by-pass AAV rep gene regulation by substituting the p5 promoter with the SV40 early promoter failed (Labow et al., 1988, Journal of Virology 62:1705-1712). Instead of constitutive Rep expression, the 15 heterologous promoter unexpectedly behaved in the same manner as the endogenous p5 promoter; repressed in the absence and activated in the presence of Ad (Labow et al., 1988, Journal of Virology 62:1750-1712). While these studies were the first to suggest rep repression as a mechanism for regulating 20 heterologous promoters, these findings also implied that AAV p5 products may be a rate-limiting factor in AAV production (Labow et al., 1988, Journal of Virology 62:1705-1712). Further efforts in this area have suggested that overexpression of Rep may increase rAAV vector yields (Flotte 25 et al., 1995, Gene Therapy 2:29:37).

An essential feature for use of rAAV as an efficient delivery system is the ability to produce recombinant stocks of virus. Although rAAV titres can approach wild type (wt) levels after multiple rounds of purification and 30 concentration, the overall total yield is still substantially lower than that of wild type AAV. Therefore, methods that increase the ability to produce high titre rAAV viral stocks will facilitate the use of rAAV delivery systems in gene therapy.

35

3. SUMMARY OF THE INVENTION

The present invention provides methods and compositions for increasing the production of high titre stocks of recombinant AAV. The invention is based on the discovery that decreased expression of AAV REP proteins results in increased synthesis of viral capsid proteins and replication of viral DNA resulting in production of high titre recombinant viral stocks. Such recombinant AAV stocks may be used in gene therapy for the purpose of transferring genetic information into appropriate host cells for the management and correction of human disease including inherited and acquired disorders such as cancer and AIDS.

The invention encompasses methods for increasing the production of high titre stocks of recombinant AAV by regulating the expression levels and/or activity of the AAV REP proteins in a host cell. The invention further encompasses compositions such as recombinant helper plasmids that are genetically engineered to express low levels of biologically functional viral REP proteins. In such helper plasmids the expression of REP proteins may be regulated at the transcriptional, translational and/or post-translational level.

The expression of REP proteins may be regulated at the transcriptional level through the use of tightly controlled promoter systems that result in either low level, or inducible, expression of the REP gene. Such promoters can be genetically engineered into recombinant helper plasmids that are designed to express low levels of REP protein. Further, triple helix molecules can be utilized to reduce the level of REP gene expression. Such triple helix molecules can be designed to hybridize to the promoter region of the REP gene and thereby inhibit REP gene transcription.

Further, the invention encompasses the coding region of the REP genes which are genetically engineered to replace the initiator MET codon with a less efficiently translated initiator codon. The genes encoding the viral REP proteins of the present invention can also be genetically engineered to contain specific 5' nucleotide sequences to which

translation repressor proteins bind. The binding of such repressor proteins to the 5' end of the REP mRNA molecules will result in inhibition of REP mRNA translation. Using such a system the level of REP protein can be controlled by
5 regulating the level and/or activity of the translational repressor protein in the host cell.

Alternatively, the level of REP expression may also be controlled altering the stability of REP mRNA. For example, the half life of the REP mRNAs may be significantly decreased
10 by genetically engineering nucleotide sequences rich in A and U nucleotides in the 3' untranslated region (UTR).

Additionally, REP mRNAs containing recognition sites in their 3' UTR for specific endonucleases may be generated using recombinant DNA techniques.

15 The level of REP protein may be controlled by taking advantage of a translational process referred to as translational recoding. In such a process, a specific recording signal in the mRNA molecule causes the growing polypeptide chain occasionally to slip backward by one
20 nucleotide on the ribosome as translation proceeds, causing the mRNA to be read in the incorrect reading frame.

The level of REP protein expressed in a host cell may further be reduced through the use of antisense and ribozyme molecules. Antisense approaches involve the design of
25 oligonucleotides that bind to the complementary REP RNA and suppress translation of REP RNA. Ribozymes molecules may be designed that include one or more sequences complementary to REP RNA and which function to specifically and efficiently catalyses endonucleolytic cleavage of REP RNA sequences.

30 Finally, mutant forms of the REP proteins may be generated that have decreased activity and/or decreased protein stability. The activity of the REP proteins may be regulated through the use of temperature sensitive REP mutants. Alternatively, REP proteins which are less stable,
35 i.e., REP proteins that possess a shorter half-life or REP proteins that are more susceptible to proteolytic cleavage,

may be utilized as a means for decreasing the activity of the REP proteins.

The invention is demonstrated by way of examples, in which the overexpression of the REP gene was shown to inhibit 5 rAAV DNA replication and CAP gene expression. In contrast, reduced production of AAV REP protein expression was sufficient for production of higher titres of recombinant virus.

10 4. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Construction of AAV helper plasmids. Plasmids AAV/AD and ACG-2 contain the endogenous p5 promoter (depicted in open boxes), while plasmids CMV/AAV, HIV/AAV and SV/AAV contain the heterologous promoters (depicted in hatched 15 boxes) replacing the original p5 promoter. All the constructs contain the same AAV coding sequences, i.e. the Rep and Cap genes diagrammed in shaded boxes, except that construct ACG-2 has an ATC to ACG mutation at the translation initiation codon of Rep.

20

Figure 2. Western analysis of Rep gene expression from various AAV helper plasmids. 293 cells were transfected with plasmid AAV/AD (lanes 1 & 6), ACG-2 (lanes 2 & 7), CMV/AAV (lanes 3 & 8), HIV/AAV (lanes 4 & 9) and SV/AAV (lanes 5 & 25 10) in the absence of Ad infection (lanes 1 through 5) or in the presence of Ad infection (lanes 6 through 10). Samples of cell lysates were separated by 10% PAGE. Western blot was performed with an anti-Rep monoclonal antibody, which recognizes all four Rep proteins (Hunter et al., 1992, J. 30 Virology 66:317-324).

Figure 3. Southern analysis of rAAV DNA replication. AAV vector plasmid pdx31-LacZ were cotransfected into 293 cells with helper plasmids CVM/AAV (lanes 1 through 4) or 35 HIV/AAV (lanes 5 through 8) in the absence of adenovirus infection (lanes 1 & 2, 5 & 6) or in the presence of adenovirus infection (lanes 3 & 4, 7 & 8). Low molecular

weight DNA were recovered from the cells and separated on 1% agarose gel without prior DpnI digestion (lanes 1, 3, 5 & 7) or with DpnI digestion (lanes 2, 4, 6 & 8). Southern blot was performed with a ³²P labeled LacZ probe (a 2.1 kb 5 ClaI/NdeI fragment).

Figure 4. Comparison of rAAV DNA replication in cells transfected with different helper plasmids. AAV vector plasmid pdx31-LacZ were cotransfected into 293 cells with 10 helper plasmids CMV/AAV (lanes 1 & 2), HIV/AAV (lanes 3 & 4), SV/AAV (lanes 5 & 6), AAV/AD (lanes 7 & 8) and ACG-2 (lanes 9 & 10) in the presence of Ad infection. Low molecular weight DNA was recovered from the cells post transfection 24 hours (lanes 1, 3, 5, 7 and 9) or 48 hours (lanes 2, 4, 6, 8 and 10). Southern blot was performed with a ³²p, labeled LacZ probe (a 2.1 kb Cla I/Nde I fragment).

Figure 5. Western analysis of Cap gene expression from different AAV helper plasmids. AAV vector plasmid pdx31-LacZ 20 were cotransfected into 293 cells with helper plasmids CMV/AAV (lanes 1 & 6), HIV/AAV (lanes 2 & 7), SV/AAV (lanes 3 & 8), AAV/AD (lanes 4 & 9) and ACG-2 (lanes 5 & 10) in the presence of Ad infection (lanes 1 through 5) or in the absence of Ad infection (lanes 6 through 10). Samples of 25 cell lysates were separated by 10% PAGE. Western blot was performed with an anti-Cap polyclonal antibody which recognizes all three Cap proteins.

5. DETAILED DESCRIPTION OF THE INVENTION

30 The present invention relates to methods for increasing the production of high titre stocks of recombinant AAV (rAAV) through regulation of expression of the AAV REP proteins. To generate recombinant viral stocks, a recombinant vector containing a gene of interest and the cis-required AAV 35 terminal repeat sequences is transfected into a host cell that is capable of providing helper virus function, and supplying in trans AAV REP and CAP proteins. The methods of

the invention are based on the observation that low level in trans expression of the AAV REP proteins increases the efficiency of rAAV DNA replication and the production of AAV viral capsid protein resulting in production of higher titre 5 recombinant viral stocks.

In particular, the invention described in the subsections below encompasses methods for regulating the level of REP protein expression. The expression of the REP proteins may be regulated at either the transcriptional or 10 translational level. The invention relates to recombinant helper plasmids that are genetically engineered to express low levels of AAV viral REP protein. The expression of REP protein may be regulated at the level of transcription through the use of tightly controlled promoter systems. 15 Alternatively, triple helix molecules may be utilized to interfere with the transcription of the REP genes.

The invention also encompasses the regulation of REP protein expression at the translational level through the use of antisense and ribozyme molecules. Further, the coding 20 region of the REP genes may be genetically engineered to replace the initiator MET codon with a less efficiently translated initiator codon. In yet another embodiment of the invention, the level of REP protein may be regulated through genetic engineering of specific nucleotide sequences into the 25 REP genes that function to decrease REP mRNA stability. Alternatively, nucleotide bind sites for translational repressor proteins may be genetically engineered into the REP gene sequences.

The present invention is based on the observation that 30 when a series of AAV helper plasmids containing various strong heterologous promoters substituted for the AAV p5 promoter were transfected into human 293 cells, less efficient rAAV DNA replication and lower capsid protein synthesis was observed. In contrast, a novel construct which 35 reduced REP expression, resulted in a 8 fold increase in rAAV yield. These observations indicate that unregulated overexpression of REP proteins adversely affects rAAV

production and suggest a role for highly regulated REP gene expression in optimal rAAV production.

5.1. RECOMBINANT EXPRESSION OF AAV REP PROTEIN

5 The present invention encompasses recombinant helper plasmids that are genetically engineered to provide *in trans* low level expression of viral REP protein. In accordance with the invention the open reading frame which encodes the AAV REP proteins may be engineered into expression vectors
10 for highly regulated expression of the REP proteins. In order to express REP proteins, the nucleotide sequence coding for the REP proteins, or a functional equivalent, is inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for the highly regulated
15 transcription and translation of the inserted REP coding sequences.

Methods which are well known to those skilled in the art can be used to construct expression vectors containing the REP protein coding sequences operatively associated with
20 appropriate transcriptional/translational control signals for highly regulated expression of REP. These methods include in vitro recombinant DNA techniques, synthetic techniques, and in vivo recombination/genetic recombination. See, for example, the techniques and vectors described in Maniatis, et
25 al., 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates & Wiley Interscience, N.Y.

The sequences of the AAV REP genes are reported in
30 Srivastava, A., et al., 1983, J. Virol. 45:555-564; Muzyczka, N., 1992, Curr. Top. Micro Immunol. 158:97-129, and Ruffing, M., et al., 1992, J. Virol. 66:6922-6930, 1992. Sources for the AAV REP genes may include the mammalian virus serotypes AAV-1, AAV-2, AAV-3, AAV-4, and AAV-5, as well as bovine AAV
35 and avian AAV. The invention contemplates, in addition to the REP DNA sequences disclosed therein, (1) any DNA sequence that encodes the same amino acid sequence for REP shown in

Srivastava, A., et al., supra; Muzyczka, N., supra and Ruffing, M., et al. supra; (2) any DNA sequence that hybridizes to the complement of the coding sequences disclosed therein under highly stringent conditions, e.g.,
5 washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel F.M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3) and encodes a functionally equivalent gene product; and/or 3) any DNA sequence that
10 hybridizes to the complement of the coding sequences disclosed therein under less stringent conditions, such as moderately stringent conditions, e.g., washing in 0.2xSSC/0.1% SDS at 42°C (Ausubel et al., 1989, supra), yet which still encodes a functionally equivalent gene product.
15 Nucleic acids which encode derivatives (including fragments) and analogs of native REP proteins can also be used in the present invention, as long as such derivatives and analogs retain the ability to provide the functions required for AAV DNA replication. In particular, REP
20 derivatives can be made by altering REP sequences by substitutions, additions, or deletions that provide for functionally active molecules that may have a altered phenotype. Furthermore, due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode
25 substantially the same or a functionally equivalent AAV REP amino acid sequence may be used in the practice of the methods of the invention. The gene product may contain deletions, additions or substitutions of amino acid residues within the sequence which result in silent changes thus
30 producing a bioactive product. Such amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amipathic nature of the residues involved. For example, negatively charged amino acids include aspartic acid and
35 glutamic acid; positively charged amino acids include lysine and arginine; amino acids with uncharged polar head groups or nonpolar head groups having similar hydrophilicity values

include the following: leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, tyrosine.

In addition, nucleic acids which encode derivatives and
5 analogs of native REP proteins with altered phenotypes may also be used in the present invention. In particular, alterations that decrease the biological activity of the AAV REP proteins and/or decrease the stability of the AAV REP
10 encoding temperature sensitive mutants of the REP protein may be genetically engineered into recombinant helper plasmids. Alternatively, nucleic acid molecules encoding REP proteins having a shorten half life, or REP proteins which are more susceptible to proteolytic cleavage may be engineered into
15 recombinant helper plasmids.

A variety of host-expression vector systems may be utilized to express the AAV REP proteins. The expression systems that may be used for purposes of the invention include but are not limited to mammalian cell systems (e.g.,
20 COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter). Promoters to express the REP
25 proteins within a cell line may be drawn from those that are highly regulated within the host cell. Inducible gene regulation may be achieved using simple inducible promoter systems, including but not limited to, the metallothionein promoter (MT) and heat shock promoter, or by using the mouse
30 mammary tumor virus promoter (MMTV) which is responsive to glucicorticoid stimulation. Alternatively, a more flexible though more complex inducible regulation system can be achieved through a "binary" gene approach. These binary regulation systems utilize a transactivator gene product to
35 control expression of a second gene of interest. In addition, repressor based binary systems may be used to regulate gene expression (Brown et al., 1987, Cell 48:555-566; Figge et

al., 1988, Cell 49:603-612). For example, the tetR system utilizes the bacterial repressor tetR and insertion of tetR operator sequences in the promoter region of a gene of interest. Induction of gene expression in such a system involves the application of an inducer molecule that binds to and inactivates the repressor molecule resulting in activation of gene expression.

The REP coding region may be linked to any number of promoters in an expression vector that can be activated in the chosen cell line. Additionally, this cassette (REP genes and promoter) is carried by a vector that contains a selectable marker so that cells receiving the vector may be identified. Selectable markers and their attendant selection agents can be drawn from the group including but not limited to aminoglycoside phosphotransferase/G418, hygromycin-B phosphotransferase/hygromycin-B, and amplifiable selection markers such as dihydrofolate reductase/methotrexate and others known to skilled practitioners.

Specific initiation signals are also required for sufficient translation of inserted protein coding sequences. These signals include the ATG initiation codon and adjacent sequences. These exogenous translational control signals and initiation sequences can be of a variety of origins, both natural and synthetic. For example, *E. coli* expression vectors will contain translational control sequences, such as an appropriately positioned ribosome binding site and initiation ATG.

Expression of the viral REP protein may be controlled at the level of translation by the replacement of the REP protein ATG start codon with the less efficient ACG codon resulting in a decrease in the production of REP protein. In a specific embodiment of the invention, a recombinant helper plasmid was constructed to contain an ATG to ACG mutation in the start codon of REP. When such a construct was utilized to generate rAAV stocks, the average yield of rAAV was increased by at least 8-fold when compared to plasmids expressing wild type levels of AAV REP protein.

In addition, the 5' end of the viral REP gene may be genetically engineered to contain specific nucleotide sequences to which translation repressor proteins bind (Melefors, 1993, Bioessays 15:85-90). Binding of the translation repressor protein to a mRNA molecule decreases the translation of the mRNA. Using such a system, the level of REP protein maybe controlled by regulating the level or activity of the translational repressor protein. Such sequences include, but are not limited to, sequences such as the iron-response element. The iron response element folds into a stem-loop structure that binds a translation repressor protein called aconitase which blocks the translation of any RNA sequence downstream. Aconitase is an iron-binding protein, and exposure of the cell to iron causes it to dissociate from the RNA, releasing the block to translation. Therefore, modification of the 5' end of the REP gene to include the iron responsive element provides a system for selectively and efficiently inducing the expression of the REP protein by exposing cells to iron.

The translation of REP mRNA may also be controlled taking advantage of a translational process referred to as translational recoding. In such a process, a specific recoding signal in the mRNA molecule causes the growing polypeptide chain occasionally to slip backward by one nucleotide on the ribosome as translation proceeds. The ribosome then resumes translation in a new reading frame resulting in production of a truncated protein. In an embodiment of the invention, a recoding signal sequence, which consists of the nucleotides UUUUUUA, may be included in AAV REP encoding recombinant helper plasmids to produce the desired low level expression of AAV REP protein.

The level of REP expression may also be controlled by altering the stability of REP mRNA. More specifically, the half life of the REP mRNA may be significantly decreased by including specific sequences known to stimulate RNA degradation in the REP gene. For example, the half-life of the REP mRNA may be significantly decreased by cloning of a

sequence rich in A and U nucleotides in the 3' untranslated (UTR) of the REP gene. This AU-rich sequence accelerates mRNA degradation. In addition, REP mRNAs containing recognition sites in their 3' UTR for specific endonucleases 5 that cleave RNA may be generated using recombinant DNA techniques. The use of such sequences will result in low level expression of AAV REP protein.

5.2. ANTISENSE AND RIBOZYME BASED REGULATION OF REP PROTEIN EXPRESSION

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In another embodiment of the invention, antisense and ribozyme molecules which inhibit expression of the REP gene can also be used in accordance with the invention to reduce the level of REP gene expression, thus effectively reducing the level of REP gene activity. Still further, triple helix 15 molecules can be utilized in reducing the level of REP gene activity. Such techniques are described below.

Antisense approaches involve the design of oligonucleotides (either DNA or RNA) that are complementary to REP gene mRNA. The antisense oligonucleotides will bind 20 to the complementary REP gene mRNA transcripts and prevent translation. Absolute complementarity, although preferred, is not required. A sequence "complementary" to a portion of an RNA, as referred to herein, means a sequence having 25 sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double-stranded antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of 30 complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by 35 use of standard procedures to determine the melting point of the hybridized complex.

Oligonucleotides that are complementary to the 5' end of the message, e.g., the 5' untranslated sequence up to and including the AUG initiation codon, should work most efficiently at inhibiting translation. However, sequences complementary to the 3' untranslated sequences of mRNAs have recently shown to be effective at inhibiting translation of mRNAs as well. See generally, Wagner, R.; 1994, Nature 372:333-335. Thus, oligonucleotides complementary to either the 5'- or 3'- non-translated, non-coding regions of REP could be used in an antisense approach to inhibit translation of REP mRNA. Oligonucleotides complementary to the 5' untranslated region of the mRNA should include the complement of the AUG start codon. Antisense oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation but could be used in accordance with the invention. Whether designed to hybridize to the 5', 3'- or coding region of REP gene mRNA, antisense nucleic acids should be at least six nucleotides in length, and are preferably oligonucleotides ranging from 6 to about 50 nucleotides in length. In specific aspects, the oligonucleotide is at least 10 nucleotides, at least 17 nucleotides, at least 25 nucleotides or at least 50 nucleotides.

Regardless of the choice of REP sequence, it is preferred that *in vitro* studies are first performed to quantitate the ability of the antisense oligonucleotide to inhibit gene expression. It is preferred that these studies utilize controls that distinguish between antisense gene inhibition and nonspecific biological effects of oligonucleotides. It is also preferred that these studies compare levels of the REP RNA or protein with that of an internal control RNA or protein. Additionally, it is envisioned that results obtained using the antisense oligonucleotide are compared with those obtained using a control oligonucleotide. It is preferred that the control oligonucleotide is of approximately the same length as the test oligonucleotide and that the nucleotide sequence of the

oligonucleotide differs from the antisense sequence no more than is necessary to prevent specific hybridization to the REP sequence.

The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, hybridization, etc. The oligonucleotide may include other appended groups such as peptides (e.g., for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (see, e.g., Letsinger et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:6553-6556; Lemaitre et al., 1987, Proc. Natl. Acad. Sci. 84:648-652; PCT Publication No. WO88/09810, published December 15, 1988) or the blood-brain barrier (see, e.g., PCT Publication No. WO89/10134, published April 25, 1988), hybridization-triggered cleavage agents. (See, e.g., Krol et al., 1988, BioTechniques 6:958-976) or intercalating agents. (See, e.g., Zon, 1988, Pharm. Res. 5:539-549). To this end, the oligonucleotide may be conjugated to another molecule, e.g., a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

The antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine,

uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

The antisense oligonucleotide may also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the antisense oligonucleotide comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the antisense oligonucleotide is an α -anomeric oligonucleotide. An α -anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β -units, the strands run parallel to each other (Gautier et al., 1987, Nucl. Acids Res. 15:6625-6641). The oligonucleotide is a 2'-O-methylribonucleotide (Inoue et al., 1987, Nucl. Acids Res. 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue et al., 1987, FEBS Lett. 215:327-330).

Oligonucleotides of the invention may be synthesized by standard methods known in the art, e.g. by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein et al. (1988, Nucl. Acids Res. 16:3209), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451), etc.

A preferred method of delivery utilizes a recombinant DNA construct in which the antisense oligonucleotide is placed under the control of a strong pol III or pol II

promoter. The use of such a construct to transfect host cells will result in the transcription of sufficient amounts of single stranded RNAs that will form complementary base pairs with the REP gene transcripts and thereby prevent translation of the REP or pathway gene mRNA. For example, a vector can be introduced in vivo such that it is taken up by a cell and directs the transcription of an antisense RNA. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence encoding the antisense RNA can be by any promoter known in the art to act in mammalian, preferably human cells. Such promoters can be inducible or constitutive. Such promoters include but are not limited to: the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42), etc. Any type of plasmid, cosmid, YAC or viral vector can be used to prepare the recombinant DNA construct which can be introduced directly into the tissue site. Alternatively, viral vectors can be used which selectively infect the desired tissue.

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA (For a review see, for example Rossi, J., 1994, Current Biology 4:469-471). The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary REP RNA, followed by an endonucleolytic cleavage. The composition of ribozyme molecules must include one or more sequences complementary to the REP gene mRNA, and must include the well known catalytic sequence responsible for mRNA cleavage. For

this sequence, see U.S. Pat. No. 5,093,246, which is incorporated by reference herein in its entirety. As such, within the scope of the invention are engineered hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of RNA sequences encoding REP gene proteins. Ribozyme molecules designed to catalytically cleave REP gene mRNA transcripts can also be used to prevent translation of REP gene mRNA and expression of REP gene. (See, e.g., PCT International Publication WO90/11364, published October 4, 1990; Sarver et al., 1990, Science 247:1222-1225). While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy REP gene mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the REP mRNA. The sole requirement is that the REP mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Haseloff and Gerlach, 1988, Nature, 334:585-591. Preferably the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of the REP mRNA; i.e., to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

The ribozymes of the present invention also include RNA endoribonucleases (hereinafter "Cech-type ribozymes") such as the one which occurs naturally in Tetrahymena Thermophila (known as the IVS, or L-19 IVS RNA) and which has been extensively described by Thomas Cech and collaborators (Zaug, et al., 1984, Science, 224:574-578; Zaug and Cech, 1986, Science, 231:470-475; Zaug, et al., 1986, Nature, 324:429-433; published International patent application No. WO 88/04300 by University Patents Inc.; Been and Cech, 1986, Cell, 47:207-216). The Cech-type ribozymes have an eight base pair active site which hybridizes to a REP RNA sequence whereafter cleavage of the REP RNA takes place. The invention encompasses those Cech-type ribozymes which REP

eight base-pair active site sequences that are present in REP or pathway gene.

As in the antisense approach, the ribozymes can be composed of modified oligonucleotides (e.g. for improved 5 stability, targeting, etc.) and should be delivered to cells which express the REP gene *in vivo*. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive pol III or pol II promoter, so that transfected cells will produce 10 sufficient quantities of the ribozyme to destroy endogenous REP gene messages and inhibit translation. Because ribozymes unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

Anti-sense RNA and DNA, ribozyme, and triple helix 15 molecules of the invention can be prepared by any method known in the art for the synthesis of DNA and RNA molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides and oligoribonucleotides well known in the art such as for example solid phase phosphoramidite 20 chemical synthesis. Alternatively, RNA molecules can be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences can be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as 25 the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

Various well-known modifications to the DNA molecules 30 can be introduced as a means of increasing intracellular stability and half-life. Possible modifications include, but are not limited to, the addition of flanking sequences of ribo- or deoxy- nucleotides to the 5' and/or 3' ends of the molecule or the use of phosphorothioate or 2' O-methyl rather 35 than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone.

5.3. CELL LINES ENGINEERED TO EXPRESS LOW LEVELS OF REP PROTEIN

Cell lines may be engineered that will natively express low levels of the AAV REP proteins. To engineer an AAV REP producing cell line, cells can be transfected with recombinant helper plasmids vector into which the AAV REP open reading frame has been inserted. Standard recombinant DNA techniques may be used to construct the recombinant vectors using the methods described above in Section 5.1. (Ausubel, F. et al., eds., Current Protocols in Molecular Biology, Wiley & Sons, New York, 1994). Transfection may be accomplished with any of the standard techniques in the art. Alternatively, a cell line can be established with the use of viral vectors that are capable of integrating DNA into the host cell genome. Examples of these vectors include those derived from retroviruses or AAV.

Cell lines which may be chosen for integration include but are not limited to HeLa, COS, NIH 3T3, and others well known to those skilled in the art. The REP coding region may be linked to any number of heterologous promoters that can be activated in the chosen cell line. Additionally, this insertion cassette (REP genes and promoter) may be linked to a gene coding for a selectable marker, in which case the integration of the REP coding region with the linked marker will confer the particular phenotype afforded by the marker to a stably transfected cell. Thus, the cells that have successfully integrated the REP genes will be selectable. Alternatively, the selectable marker may be transfected on a separate plasmid.

Promoters to express the REP proteins within a cell line may be drawn from those that are functionally active within the host cell. Such promoters, which are well known in the art, will include those promoters that are highly regulated within the host cell resulting in low level expression of the viral REP proteins. Inducible promoters may be also be used in the practice of the invention, including but not limited to, the metallothionine promoter (MT), the mouse mammary

tumor virus promoter (MMTV), and others known to those skilled in the art.

Selectable markers and their attendant selection agents can be drawn from the group including but not limited to 5 aminoglycoside phosphotransferase/G418, hygromycin-B phosphotransferase/hygromycin-B, and amplifiable selection markers such as dihydrofolate reductase/methotrexate and others known to skilled practitioners.

Stable expressing cell lines may also be constructed by 10 linking the AAV ITR sequence to an expression cassette containing the REP coding region with the appropriate transcriptional signals to allow for integration into the host cell genome.

Detection of the expression of the REP genes can be 15 performed by standard techniques including Northern analysis, immunoblotting, and immunoprecipitation. Such techniques may be utilized to identify cells that express low levels of REP protein.

5.4. PRODUCTION OF RECOMBINANT VIRUS STOCKS

20 The present invention relates to methods for efficient production of high titre stocks of rAAV through regulation of expression of the AAV REP proteins. The methods of the invention comprised culturing a eukaryotic cell containing helper virus, recombinant DNA encoding AAV CAP and REP 25 protein, and a recombinant nucleic acid containing a DNA sequence of interest and the required *cis*-acting AAV terminal repeat structures.

A primary goal of the present invention is to provide methods for expressing *in trans* low levels of REP protein or 30 to express REP protein with decreased biological activity and/or reduced half-life. The methods of the invention are based on the observation that reduced expression or activity of the REP protein results in production of high titre stocks of rAAV.

35 To generate recombinant viral stocks, the recombinant nucleic acid containing the DNA sequence of interest may be transfected into a host cell line that is capable of

providing helper virus function, and supplying in trans AAV REP and CAP proteins. The REP and CAP proteins are required for replication and encapsidation of the linear recombinant nucleic acid into mature viral particles.

5 The REP and CAP proteins may be supplied in trans by transfection of the host cell line with a recombinant plasmid that is capable of coding for each of the proteins. DNA transfections may be carried out using methods well known to those skilled in the art. These may include DNA transfection
10 by lipofection, electroporation or calcium phosphate precipitation (Ausubel, et al., 1989, in Current Protocols for Molecular Biology, Green Publishing Associates, Inc. and John Wiley & Sons, Inc., New York). The plasmid is transfected into the host cell line with the intention of
15 either transiently or stably expressing the REP and CAP proteins.

In addition to expressing the viral REP and CAP proteins, the host cell lines must be able to provide helper virus function. Both adenovirus and herpes simplex virus may
20 serve as helper viruses for replication and encapsidation of DNA fragments containing the cis-required AAV terminal repeat sequences. Any host cell permissive for infection by either of these two viruses or any virus that acts as a helper virus for AAV, may be used in the practice of the invention. The
25 multiplicity of infection (MOI) and the duration of the infection time will depend on the type of virus used and the cell line employed and such techniques are well known to those skilled in the art.

30 5.5. USES OF RECOMBINANT AAV VIRAL STOCKS

The rAAV viral stocks may be used in gene therapy for the purpose of transferring genetic information into appropriate host cells for the management and correction of human diseases including inherited and acquired disorders
35 such as cancer and AIDS. The rAAV can be administered to a patient at therapeutically effective doses. A therapeutically effective dose refers to that amount of the

compound sufficient to result in amelioration of symptoms of disease.

Toxicity and therapeutic efficacy of the rAAV can be determined by standard pharmaceutical procedures in cell
5 cultures or experimental animals, e.g., for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as
10 the ratio LD_{50}/ED_{50} . Doses which exhibit large therapeutic indices are preferred. While doses that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets rAAV to the site of treatment in order to minimize damage to untreated cells and reduce side
15 effects.

The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such rAAV lies preferably within a range of circulating concentrations that include the ED_{50} with
20 little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be
25 formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (i.e., the concentration of the test compound which achieves a half-maximal infection or a half-maximal inhibition) as determined in cell culture. Such information can be used to more
30 accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

Pharmaceutical compositions comprising the rAAV for use in accordance with the present invention, may be formulated
35 in conventional manner using one or more physiologically acceptable carriers or excipients. For example, the rAAV may

be suspended in a carrier such as PBS (phosphate buffered saline).

The rAAV and their physiologically acceptable salts and solvates may be formulated for administration by inhalation 5 or insufflation (either through the mouth or the nose) or for oral, buccal, parenteral or rectal administration.

For administration by inhalation, the rAAV for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized 10 packs or a nebulizer, with the use of a suitable propellant, e.g. dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered 15 amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of a therapeutic compound and a suitable powder base such as lactose or starch.

For oral administration, the pharmaceutical compositions 20 may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, 25 microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g. magnesium stearate, talc or silica); disintegrants (e.g. potato starch or sodium starch glycolate); or wetting agents (e.g. sodium lauryl sulphate). The tablets may be coated by methods well known in the art.

30 Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with 35 pharmaceutically acceptable additives such as suspending agents (e.g. sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g. lecithin

or acacia); non-aqueous vehicles (e.g. almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (e.g. methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound. For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

The rAAV may be formulated for parenteral administration by injection e.g. by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form e.g. in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

In addition to the formulations described previously, the rAAV may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example, subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the therapeutic compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

6. EXAMPLE: LOW LEVEL EXPRESSION OF AAV REP INCREASES
THE EFFICIENCY OF RECOMBINANT VIRAL PRODUCTION

The experimental results described below demonstrate that low level expression of AAV REP proteins increases the efficiency of recombinant AAV viral production.

6.1. MATERIALS AND METHODS

6.1.1. AAV HELPER PLASMIDS CONSTRUCTION

Various AAV helper plasmids were constructed following standard protocols. AAV/AD is a previously published AAV packaging plasmid containing the entire AAV coding sequences including promoter p5 and has a MW of -8.2 kb (Samulski et al., 1989, Cold Spring Harbor Lab. Press). Plasmid CMV/AAV also 8.2kb contains the entire AAV coding sequences except that the AAV p5 promoter was substituted by a cytomegalovirus immediate early promoter(CMV). Plasmid pSV/AAV is a construct similar to pCMV/AAV except that an SV40 late promoter was substituted for AAV p5 promoter. This plasmid is 7.8 kb in size. Plasmid HIV/AAV also contains the entire AAV coding sequences except the AAV p5 promoter was substituted by an HIV long terminal repeat promoter. The construct was made by three fragment ligation. The first fragment was the SspI-HindIII fragment from pHIV-Rep (Antoni et al., 1991, Journal of Virology 65:396-404) containing the complete HIV promoter and portion of the Rep gene. The second fragment was the HindIII-SnaBI fragment from psub201 (Samulski et al., 1988, Journal of Virology 62:206-210) containing the rest of the Rep gene and the entire Cap gene along with the polyadenylation site. The third fragment was the SspI-SmaBI fragment from Bluescript KS(+) (Stratagene) containing the plasmid origin and Amp^r gene. This construct is 7.6.- kb in size. Plasmid ACG-2 is a variant of AAV/AD containing an ATG to ACG mutation in the start codon of Rep [50], which reduces Rep protein synthesis, and is identical in size as the parental plasmid pAAV/Ad (8.2 kb). All the constructs were characterized by restriction analyses and

5 experiments (Sambrook et al. 1989, Molecular Cloning: a laboratory manual. Cold Spring Harbour Press, CSH, 2nd edition).

10 6.1.2. VIRUSES AND CELLS

rAAV vector was generated by calcium phosphate cotransfection methods as described previously (Xiao et al., 1996, J. Virol. 70:8098-8108). Briefly, human 293 cells were passed one day before transfection in DMEM (Gibco) containing 10% FBS (Gibco) with streptomycin and penicillin. At about 80% confluence, the cells were fed with 10 ml fresh IMDM media (Gibco) containing 10% FBS without antibiotics 1 to 2 hours before transfection. Total 25 μ g of plasmid DNA (vector plus helper at various ratio) was dissolved in 1 ml of 0.25 M CaCl_2 and then quickly mixed with 1 ml of 2XHB buffer (50 mM HEPES, 280 mM NaCl and 1.5 mM Na_2HPO_4 , pH 7.12). The DNA complex was slowly added to the cells. After incubation for 8 hours, the cells were fed with fresh DMEM medium (Gibco) containing 10% FBS and antibiotics, and infected with Adenovirus 5 (dl309) at 2 m.o.i. (multiplicity of infection). Transfection efficiencies were monitored by staining a duplicate plate for β gal gene expression and counting the number of blue cells. Two day post Ad infection, the cells were harvested together with the media and 0.1 ml of 1 M Tris-Cl (pH 8.5) was added to adjust the pH of the media. Following four cycles of freeze-thaw and removal of cell debris, the rAAV viral lysate was heated at 56°C for 30 minutes to inactivate the adenovirus and stored at -20°C before use.

35 The titers of AAV-LacZ virus were determined by counting the blue cells after X-gal staining, following coinfection of 293 cells with various dilution's of the rAAV stocks and 1

m.o.i. of adenovirus dl309 for 24 hours. All experiments were done in triplicate to ensure reproducibility and average taken for final titer.

5 6.1.3. ASSAY OF rAAV DNA REPLICATION

The rAAV DNA was recovered from the transfected cells by the method of Hirt extraction (Hirt, B., 1967, J. Mol. Biol. 26:365-369) with modifications. Briefly, the cell pellet from 1/10 to 1/5 of a 10 cm dish was resuspended in 270 μ l of 10 20 mM Tris-Cl, 20 mM EDTA(pH8.0) and lysed by addition of 30 μ l of 10% SDS. The cell lysate was incubated at 37°C for 1 hour with 50 μ g/ml proteinase K and mixed with 80 μ l of 5 M NaCl. After stored on ice for more than 1 hour, the cell lysate was centrifuged at 15,000 rpm at 4°C for 30 minutes. 15 The supernatant was recovered and subsequently extracted with phenol, phenol-chloroform and chloroform. Low molecular weight DNA was precipitated with equal volume of isopropanol, rinsed with 70% ethanol specific protein bands were visualized with chemiluminescence reagent (DuPont) exposed to 20 X-ray film.

6.2. RESULTS

6.2.1. EFFICIENT REP GENE EXPRESSION FROM HETEROLOGOUS PROMOTERS IN THE ABSENCE OF ADENOVIRUS

25 In order to achieve high level Rep gene expression in an adenovirus independent manner, we have constructed a number of AAV helper plasmids containing heterologous promoters substituted for the AAV p5 sequence. The heterologous promoters were the cytomegalovirus (CMV) immediate early 30 region, HIV long terminal repeat and the SV40 late promoter (Fig. 1). These sequences are among the strongest constitutive viral promoters commonly used and should express high levels of Rep. A helper plasmid pAAV/Ad (Samulski et al., 1989, J. Virol. 63:3822-3828), which retains the 35 endogenous p5 promoter, was included in this study as a control. To determine the effect of low level Rep expression

on rAAV production, a novel plasmid pACG-2 was also constructed as described above, see Section 6.1.1. This construct is identical to pAAV/Ad except that a point mutation has converted the start codon of Rep from ATG into a less efficient ACG codon. AAV utilizes the ACG start codon for AAV Vp2 capsid production, which is expressed at low levels from the same mRNA that encodes the major capsid protein Vp3. Since this mechanism of regulation is utilized by AAV, it was expected that this point mutant would reduced Rep protein synthesis without altering AAV viral mRNA levels. The various AAV helper plasmids which carried specific promoter elements in place of the AAV p5 promoter only differed at most by 600 bp (PHIV/AAV 7.6kb) when compared to the parental plasmid p AAV/AD (8.2 kb).

Repression of AAV p5 (Labow et al., 1986, J. Virol. 60:251-258; Laughlin et al., 1982, J. Virol. 41:868-876; Pereira et al., 1997, J. Virol. In Press) and heterologous promoters such as SV40 (Labow et al., 1987, Mol. & Cell. Biology 7: 1320-1325), HIV (Antoni et al., 1991, J. Virology 65:396-404; Horer et al., 1995, J. Virol. 69:5485-5496) and CMV (Heilbronn et al., 1990, J. Virol. 64:3012-3018) by rep in the absence of ad infection was overcome by using human 293 cells. E1A gene products have been shown to transactivate AAV p5 Shi et al. 1991, Cell 67: 377-388), CW (Gorman, 1989, Virology 171:377-385) and HIV (Rice et al., 1988, Proc. Nat. Acad. Sci. 85(12):4200-4204) promoters. E1A gene products in 293 cells will counteract the repression by AAV Rep proteins, and thereby increase Rep gene expression from these various promoters in the absence of ad infection.

The various AAV constructs were transfected into 293 cells with or without Ad infection, and assayed for Rep gene expression 48 hours post transfection by Western blot analysis using anti-Rep monoclonal antibody (Hunter et al., 1992, J. Virology 66:317-324). From the experimental results shown in Fig. 2, several observations can be made. First, in the absence of adenovirus in 293 cells, Rep gene expression was detected from all five AAV constructs, (Fig. 2, lane 1-

5). Plasmids pCMV/AAV and pHIV/AAV expressed extremely high levels of Rep, whereas pSV/AAV and pAAV/Ad had modest expression (Fig. 2, compare lanes 3 & 4 with lanes 1 and 5). The lower expression from the SV40 promoter in 293 cells is most likely due to SV40 enhancer repression by E1A proteins as previously described (Gorman et al., 1989, Virology 171:377-385; Velcich et al., 1985, Cell. 40:705-716). As expected, plasmid pACG-2 generated the least amount of Rep (Fig. 2, lane 2). Second, in the presence of adenovirus, the levels of Rep appeared the same as the minus Ad extracts for pCMV/AAV, pHIV/AAV and pSV/AAV (compare Fig. 2, lanes 3, 4 & 5- with lanes 8,9 & 10). However, for pAAV/Ad and pACG-2, the Rep levels appeared slightly lower after Ad infection, implying further modulation of AAV gene regulation by Ad coinfection. Third, although Rep 52/40 expression was not altered after Ad infection, the ratio of Rep over Rep52/40 was significantly different among the various constructs (Fig.2). The helper plasmids with strong heterologous promoters, such as pCMV/AAV and pHIV/AAV, demonstrated obvious abnormal Rep vs. Rep52/40 ratios (Fig. 2, lanes 3 to 5 and 8 to 10), while pAAV/Ad expressed p5 and p19 Bioducts at roughly one to one ratio (Fig. 2, lanes 1 & 6). For pACG-2, levels were less than one (Fig. 2, lanes 2 & 7), suggesting that p19 products (Rep52/40) were made at higher levels than the p5 products (Rep).

6.2.2. EXPRESSION OF REP GENE WAS NOT SUFFICIENT TO REPLICATE rAAV DNA

Since these results demonstrate that high levels of Rep protein can be expressed independent of ad infection, rAAV DNA replication was assayed under these conditions. Plasmids pCMV and pHIV, which demonstrated highest expression of Rep, were tested in cotransfection experiments with a rAAV-LacZ vector plasmid as a replication substrate (McCown et al., 1996, Brain Research 713:99-107). As a control, the same constructs were assayed for rAAV-LacZ replication in the presence of Ad infection.

48 hours post transfection, low molecular weight DNA was recovered and analyzed by Southern blot. To distinguish between input plasmid and newly replicated DNA, samples were treated with or without Dpn I endonuclease. An autoradiography of the experiment is shown in Figure 3. Resistance to Dpn I digestion suggested that in the presence of Ad infection, Rep proteins from PCMV/AAV and PHIV/AAV successfully replicated the rAAV-LacZ vector DNA (Fig. 3; lanes 3 & 4 represent PCW/AAV and lanes 7 & 8 represent pFUV/AAV). However, sensitivity to Dpn I digestion in the absence of Aa infection (Fig. 3, lanes 1 & 2, pCNW/AAV and lanes 5 & 6, PHIV/AAV), suggested that rAAV vector DNA failed to replicate even though abundant Rep proteins were produced from these helper constructs (Fig. 2, lanes 3 & 4). Similarly, cotransfection of the rAAV-LacZ vector plasmid, with other helper plasmids, such as pAAV/Ad and PSV/AAV, in the absence of Ad infection also failed to replicate the vector DNA. These results support the conclusion that constitutive Rep gene expression is not sufficient to mediate AAV DNA replication and that other ad helper functions besides the constitutive expression of E1 in 293 cells is required.

6.2.3. OVEREXPRESSION OF REP GENE INHIBITS rAAV TITERS

The above experiments demonstrated that the new helper constructs can obtain high levels of Rep proteins which are functional for AAV replication only in the presence of Ad coinfection. To further characterize these helper constructs for rAAV production, the yield of vector particles generated after transfection experiments was measured. For example, an 1:1 ratio of rAAV vector vs. helper plasmid (pAAV/Ad) results in optimal rAAV yield when using calcium phosphate transfection method in 293 cells (Xiao et al., 1996, J. Virol. 70:8098-8108). The calcium phosphate method was used to measure the efficiency of the new helper plasmids in this experiment. Three different vector:helper ratios (3:1, 1:1

and 1:3) were tested that covered a 9 fold range. Eight hours post transfection, the media was changed and the cells were infected with Ad5 dl309 and incubated for additional 48 to 60 hours till full cytopathic effect (CPE) was observed. 5 Since approximately 10% to 30% of the rAAV viruses generated are released in the culture media, the cells were harvested together with the media before assaying for particle number. After four cycles of freeze-thaw and removal of cell debris, the lysates were heated at 56°C for 30 minutes to inactivate 10 any residual adenovirus and tittered on 293 by staining for b-gal activity (see Section 6.1).

As shown in Table 1, helper plasmids pCMV/AAV and pHIV/AAV generated the lowest rAAV yields, even though these constructs produced the highest Rdp levels. Plasmid pSV/AAV 15 resulted in rAAV yields higher than pCMV/AAV and pHIV/AAV but significantly lower than pAAV/Ad. This is an interesting observation since both of these plasmids produced similar level of Rep (see Fig. 2, compare lane 1 and 6 with lane 5 and 10). The overall yields of rAAV generated from pCW/AAV, 20 pHIV/AAV and pSV/AAV were not dramatically effected by the 9 fold range of vector:helper ratio (Table 1), suggesting that the rate-limiting factor is not merely the level of rep protein or quantity of helper plasmid, but some fundamental difference between pAAV/Ad and the other three helper 25 plasmids. As possible explanations for the decrease in vector yield, pCMV/AAV and pHIV/AAV overexpression of Rep may have inhibited other genes, both viral (AAV and/or Ad) and cellular. In addition, the substitution of the p5 promoter may have removed essential cis-acting regulatory functions 30 required for appropriate p19 and p40 expression (McCarty et al., 1991, J. Virol. 65:2936-2945).

This hypothesis is supported by the results of helper plasmid pACG-2 which produced the highest rAAV titers (Table 1). This construct retained the p5 promoter sequences but 35 reduced Rep expression through inefficient translational initiation (Fig. 1; Fig. 2, lanes 2 & 7). The average rAAV yields from this plasmid was increased by 8-fold when

compared to its parental plasmid AAV/AD. The results suggested that unregulated overexpression of Rep may have a negative effect, while lower but sufficient Rep expression can generate higher rAAV yields.

5 These observations are not in agreement with a previous report (Flotte et al., 1995, Gene Therapy 2:29-37), where an AAV helper plasmid (pRS5) containing the same HIV LTR showed a 7.5 fold increase in rAAV virus yield when compared to pAAV/Ad. In the experiments described herein the helper
10 construct pHIV/AAV gave rise to much lower rAAV yields than pAAV/Ad. One important variable which may explain this difference is the addition of adenovirus relative to transfection. In our experiments adenovirus infection was carried out 8 hours post plasmid transfection instead of 1
15 hour before as described by Flotte et al (Flotte et al., 1995, Gene Therapy 2:29-37). In an attempt to better explain these differences, this possibility was tested by infecting with Ad at one hour before, simultaneously, or 8 hours post plasmid transfection. The cells were harvested 48 hours
20 after Ad infection and the rAAV titers were measured. The results shown in Table 2 indicated that when pAAV/Ad was used as the helper plasmid less than 2-fold differences in rAAV titers were observed when the time of Ad super infection was varied (Table 2). However, the addition of Ad prior to
25 pHIV/AAV transfected plates was important (Table 2). rAAV titers were about 100 fold higher ($1.5-1.7 \times 10^7$ transducing units/10cm plate) when Ad infection was carried out one hour before or simultaneously rather than 8 hours post transfection (1.8×10^5 transducing units/10cm plate) (Table 2).
30 This implies that the presence of Ad gene expression prior to the onset of AAV Rep gene expression has a significant effect of rAAV production when using pHIV/AAV as a helper plasmid. Regardless, the best titers from pHIV/AAV were still about 10 fold lower than that of pAAV/Ad. Since pAAV/Ad contains Ad
35 ITRs flanking AAV genes, the Ad ITR sequences may influence AAV gene expression and explain the difference in vector yield observed. To examine this possibility, the AAV gene

cassette was deleted from pAAV/Ad and replaced by pHIV/AAV. This construct like pAAV/Ad now has HIV/AAV sequences flanked by the Ad ITRS. This new variant of pHIV/AAV/Ad did not produce better rAAV yields despite the addition of the Ad 5 ITRs. The discrepancy between our results and others (Flotte et al., 1995, Gene Therapy 2:29-37) can only partially be attributed to the difference in the helper plasmids. However, based on the results observed concerning the time of addition for Ad infection with pHIV/AAV, this may be the rate 10 limiting factor which influences rAAV yields when using strong constitutive promoters such as the HIV LTR. In addition, one may need to consider the numerous variables utilized during rAAV production, such as different lots of 293 cells, Ad strain, various transfection and infection 15 methods, in order to make direct comparisons.

6.2.4. OVEREXPRESSION OF REP GENE INHIBITED rAAV DNA REPLICATION AND CAP GENE EXPRESSION

To explore why overexpression of Rep resulted in lower 20 rAAV particles, two essential steps were examined, viral DNA replication and capsid protein synthesis. To examine rAAV replication, the rAAV-LacZ plasmid was cotransfected with various AAV helper plasmids followed by adenovirus infection. At 24 and 48 hours post transfection, low molecular weight 25 DNA was isolated and DpnI digested, followed by Southern analysis using 32p labeled LacZ probe. The experimental result indicated that rAAV DNA replication was significantly lower when pCMV/AAV (Fig. 4, lanes 1 & 2) and pHIV/AAV (Fig. 4, lanes 3 & 4) where used as helper plasmids, as compared to 30 pSV/AAV, pAAV/Ad and pACG-2 (Fig. 4, lanes 5 through 10). PhosphoImager quantitation of the monomer bands at the 48-hour time point revealed approximately 80% reduction in vector DNA replication when comparing pCMV/AAV and PHIV/AAV to pAAV/Ad. On the other hand, no significant difference was 35 found between pSV/AAV, pAAV/Ad and pACG-2 (Fig. 4, lanes 5 through 10 and data not shown). Interestingly, rAAV DNA

replication was not reduced in pACG-2 transfected cells, although the Rep level were considerably lower (Fig. 2, lane 7). Since pCMV/AAV and pHIV/AAV expressed highest levels of Rep (Fig. 2, lanes 8 & 9) which resulted in lowest levels of rAAV DNA replication (Fig. 4, lanes 1 through 4), these data suggest that Rep are not rate-limiting factors, but may instead interfere with the vector DNA replication when overexpressed. The mechanism of inhibition remains unknown.

Since capsid proteins are essential for particle formation, the accumulation of these proteins from the various helper plasmids was also examined. 48 hour post transfection, cells were harvested and analyzed by Western blot using an anti-capsid polyclonal antibody. The results are shown in Figure 5. In the absence of Ad infection, all helper plasmids generated low but detectable level of capsid proteins, consistent with published observations that Ad infection enhances AAV capsid gene expression (Muzyczka, N., 1992, Current Topics in Microbiology & Immunology 158:97-129). In the presence of adenovirus infection, pACG-2 transfected cells synthesized and/or accumulated the highest level of capsid proteins (Fig. 5, lane 5) while pCMV/AAV and pHIV/AAV produced the lowest (Fig 5, lanes 1 & 2). From these results, a negative correlation was observed between high Rep levels (Fig. 2, pCMV/AAV lanes 8 and pHIV/AAV lane 9) and efficient capsid gene expression (Fig. 5, pCMV/AAV lanes 1 and pHIV/AAV lane 2). For pACG-2, just the reverse was observed, low rep expression (Fig. 2, lane 7) resulted in highest capsid gene expression (Fig. 5, lane 5). While pSV/AAV replicated rAAV DNA to similar levels as pAAV/AD and pACG-2 (see Fig. 4 lanes 5-10), and expressed AAV capsid gene products higher than pCMV/AAV and pHIV/AAV (Fig. 5, compare lane 3 to 1 and 2), these capsid gene products were reduced when compared to helper plasmids pAAV/Ad, and pACG-2 (Fig. 5, lane 3 vs 4 and 5). From this analysis, a direct correlation can be made between the amount of Rep protein produced, level of AAV capsid expression (Fig. 5) and the yield of rAAV (table 1) with pACG-2 expressing the lowest levels of Rep,

the highest amounts of capsid protein and the best yields of rAAV followed by pAAV/AD, pSV/AAV, pHIV/AAV and pCMV/AAV.

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TABLE 1. Comparison of rAAV titers by different helper plasmids

Helper plasmid	Vector/helper ratio	rAAV titer	
		TU/plate ^a	TU/cell ^b
CMV/AAV	3:1	2.0×10^5	0.04
	1:1	8.5×10^4	0.02
	1:3	8.5×10^4	0.02
HIV/AAV	3:1	1.4×10^5	0.03
	1:1	8.5×10^4	0.02
	1:3	1.2×10^5	0.02
SV/AAV	3:1	8.3×10^6	1.6
	1:1	6.0×10^6	1.2
	1:3	5.5×10^6	1.1
AAV/Ad	3:1	8.5×10^7	17
	1:1	1.4×10^8	28
	1:3	1.0×10^8	20
ACG-2	3:1	5.0×10^8	100
	1:1	1.1×10^9	220
	1:3	8.0×10^8	160

^a The rAAV-LacZ yields were mean values from three experiments performed with a 10-cm plate of human 293 cells. The transducing units (TU) were determined by infecting 293 cells with Ad at an MOI of 1 and various dilutions of heat-inactivated rAAV virus stocks. After X-Gal staining, each blue cell was translated into 1 TU.

^b The number of transducing units (TU) produced per cell was obtained by dividing the titers (total TU) from each 10-cm plate by the total number of 293 cells (approximately 5×10^6).

TABLE 2. Effects of Ad infection time on rAAV yields

Time of Ad infection ^a	rAAV titer (TU) ^b for:	
	AAV/Ad	HIV/AAV
Before	1.7×10^8	1.5×10^7
Same	2.5×10^8	1.7×10^7
After	1.9×10^8	1.8×10^5

^a Ad infection was carried out at three different time points, 1 h before, simultaneously with, and or 8 h after DNA transfection, for the three individual samples of a given helper plasmid.

^b The rAAV-LacZ yields were obtained from a 10-cm plate of 293 cells. The transducing units (TU) were determined by infecting 293 cells with Ad at an MOI of 1 and various dilutions of heat-inactivated rAAV virus stocks. After X-Gal staining, each blue cell was translated into 1 TU.

The present invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within 5 the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing descriptions and accompanying drawings. Such modifications are intended to fall within the scope of the 10 appended claims.

All publications and patent applications mentioned in the specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be 15 incorporated by reference.

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WHAT IS CLAIMED:

1. A method for producing a stock of recombinant adeno-associated virus comprising:

- 5 a) co-transfecting cells permissive for adeno-associated virus replication in which said cells express less than wild-type levels of AAV REP protein with i) in which said cells express less than wild-type levels of AAV REP protein; a recombinant adeno-associated virus vector which contains a foreign DNA sequence and which can be incorporated
10 into an infectious AAV virion; and ii) a recombinant helper adeno-associated virus DNA which provides viral functions sufficient for replication and packaging into infectious virions of said recombinant adeno-associated virus vector; and
15 b) collecting virions produced.

2. The method of Claim 1 wherein the level of REP protein is reduced by regulating the transcription of the REP gene.
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3. The method of Claim 1 wherein the level of REP protein is reduced by regulating the translation of the REP gene.

25 4. A host cell for producing a stock of recombinant adeno-associated virus containing a recombinant DNA vector that expresses less than wild-type levels of adeno-associated REP protein.

30 5. The host cell of Claim 4 wherein the level of REP protein is regulated at the transcriptional level.

6. The host cell of Claim 4 wherein the level of REP protein is regulated at the translational level.
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7. The host cell of Claim 6 wherein the transcription of the REP gene is under the control of the cytomeglovirus promoter.

5 8. The host cell of Claim 4 wherein the host cell is transiently transfected.

9. The host cell of Claim 4 wherein the host cell is stably transfected.

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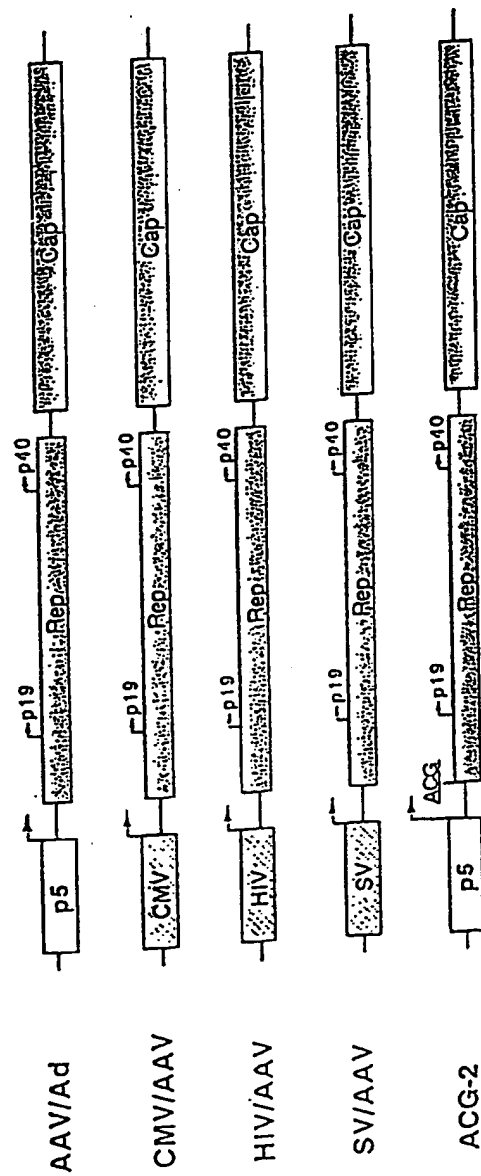


FIG. 1

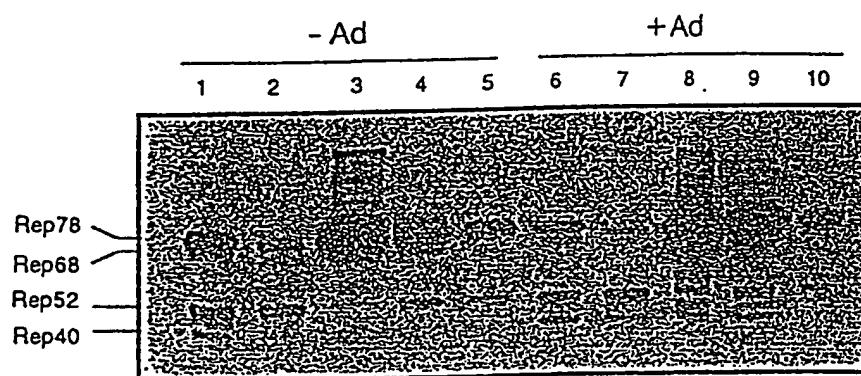


FIG. 2

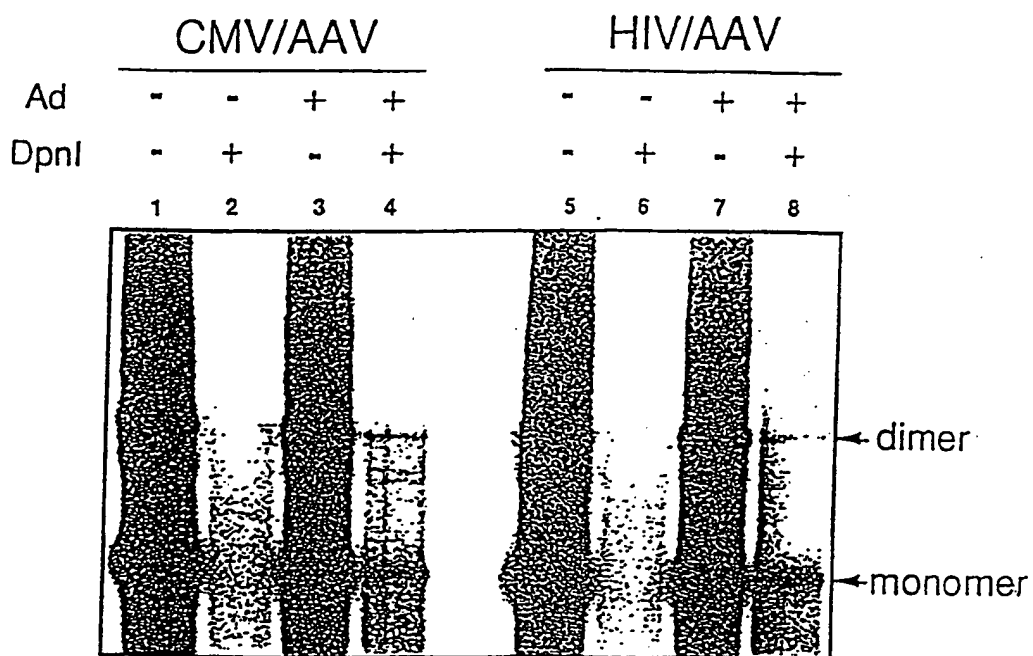


FIG. 3

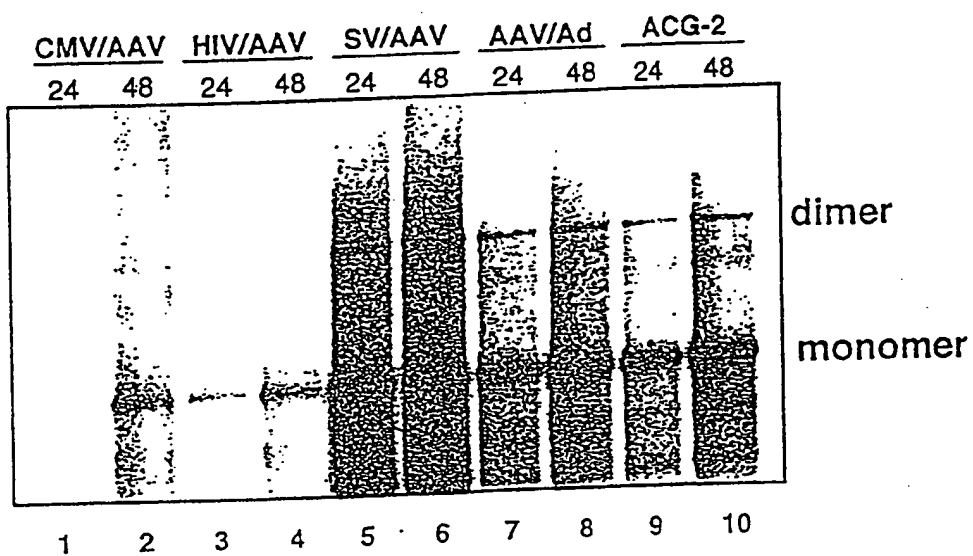


FIG. 4

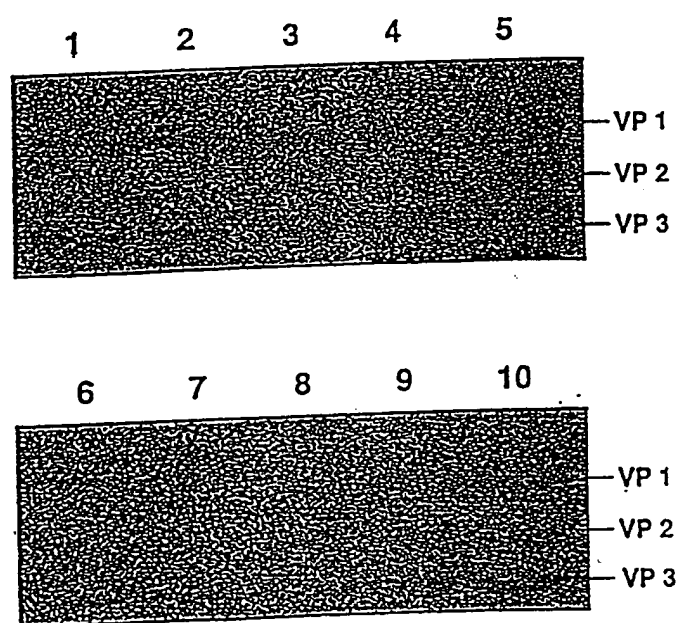


FIG. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/07654

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 5/10, 7/01, 15/10, 15/63, 15/64, 15/86

US CL : 435/172.3, 320.1, 325, 366, 369

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.3, 320.1, 325, 366, 369

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Biosis, Dialog, Biotech, Medline, Biosci

Search terms: adeno-associated virus, co-transfect, REP, CAP, transcription

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,139,941 A (MUZYCZKA et al.) 18 August 1992, columns 5-6, Fig. 1 and claims 1-4.	1-9
A	KOTIN, R.M. Prospects for the Use of Adeno-Associated Virus as a Vector for Human Gene Therapy. Human Gene Therapy. 1994, Vol. 5, pages 793-801, especially 797-799.	1-9
A	WEITZMAN et al. Adeno-Associated Virus (AAV) Rep Proteins Mediate Complex Formation Between AAV DNA and Its Integration Site in Human DNA. Proceedings of the National Academy of Sciences. June 1994, Vol. 91, pages 5808-5812, especially page 5811.	1-9

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

•	Special categories of cited documents:	•T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principles or theory underlying the invention
•A	document defining the general state of the art which is not considered to be of particular relevance		
•B	earlier document published on or after the international filing date	•X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
•L	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	•Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
•O	document referring to an oral disclosure, use, exhibition or other means		
•P	document published prior to the international filing date but later than the priority date claimed	•A	document member of the same patent family

Date of the actual completion of the international search

28 MAY 1998

Date of mailing of the international search report

08 JUL 1998

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/07654

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	YANG et al. Characterization of Cell Lines That Inducibly Express the Adeno-Associated Virus Rep Protein. Journal of Virology. August 1994, Vol. 68, No. 8, pages 4847-4856, especially 4849-4850.	1-9